ACTIVATION OF BETAGLOBIN THROUGH THE USE OF TRANSCRIPTOR ACTIVATOR-LIKE EFFECTORS TO COMBAT SICKLE CELL ANEMIA

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ACTIVATION OF BETAGLOBIN THROUGH THE USE OF TRANSCRIPTOR ACTIVATOR-LIKE EFFECTORS TO COMBAT SICKLE CELL LEUKEMIA

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LIST OF SYMBOLS AND ABBREVIATIONS

Transcriptor Activator-Like Effector	TALE
Zinc Finger Nucleases	ZFNs
Transcriptor Activator-Like Effector Nucleases	TALENs
Non-Homologous End Joining	NHEJ
Homologous Recombination	HR
Micro-RNA	mRNA

SUMMARY

The purpose of this research project is to create TALEs that will activate the betaglobin gene so that the mRNA can be detected using molecular beacons. The reason we need to upregulate or activate the gene is that during sickle cell anemia the gene is mutated. In regular cell lines the betaglobin gene is not activated, so to test our hypothesis we need to activate the gene in the cells to be able to test our gene therapies. We are using TALEs so that we can enter the nucleus, bind effector-specific DNA sequences, and transcriptionally activate gene expression. This allows us to later on use TALENs to cut the specific parts that we do not want in the DNA. This research is under the mentorship of Eli Fine in the Biomedical Engineering department and Dr. Gang Bao in the Biomedical Engineering department.

CHAPTER 1

INTRODUCTION

This research is particularly important to Sickle Cell Anemia, but also for other genetic diseases. As this is relatively new research, most of the research is more specific to the structure of TALEs and certain genes instead of actual TALEs involved in diseases. As mentioned before, the sickled cells in Sickle cell anemia occur because of mutations in the beta globin gene of the red blood cells. Our research is to upregulate or activate the beta globin gene in these cells. The reason for this is because we want the RNA of the beta globin gene. We need that RNA so that we can detect it with Molecular Beacons and find its exact location. In order to transcribe the beta globin DNA into RNA, we need to first activate or "turn on" the transcription of that gene within the cell. Once a gene is activated then we will be able to use the molecular beacons to detect the specific sequence that we want in the nucleic acid. By constructing the TALEs that can do this, we would also help further gene therapy developments of other disease models.

CHAPTER 2

BACKGROUND

The research on Transcription Activator-like Effectors has increased in recent years. The research went from Homing Endonucleases to Zinc Finger Nucleases to TALEs and TALENs. Homing Endonucleases are naturally occurring sequence-specific endonucleases that recognize and cleave long sequences in DNA (Humbert et al). Zinc Finger Nucleases are artificial proteins composed of the DNA binding domain of a zinc-finger protein fused to a Fok1 nuclease domain. TALEs are proteins that are involved in direct modulation of gene expression. TALENs on the other hand are those same proteins with a Fok1 Nuclease at the end.

Zinc Finger Nucleases are being phased out right now in favor of TALEs and TALENs. This is because Zinc Finger Nucleases are more expensive, more toxic, and not as specific as TALEs and TALENs are.

TALEs were discovered from the Xanthomonas and Ralstonia bacteria. These bacteria developed this resourceful strategy and multiplied and colonized their host plants. These bacteria are responsible for the secretion and translocation of effector proteins into the host cells. TALEs are made up of a C-terminus, which carries the nuclear localization signals, which allows the import of the protein into the nucleus of the cell. Downstream of the C-terminus lays an Activation Domain. This activation domain is involved in the recruitment of host transcriptional machinery. After that lies the series of 34 amino acid modules that are repeated in tandem. (Bodnar et al). In these amino acid modules lie repeat-variable di-residues (RVDs). Specifically at positions 12 and 13, these

RVDs allow for the variation in the TALEs. "…TALEs contain a new and unique kind of DNA-binding motif with high sequence specificity. Based on the TALE code, this remarkable feature can be exploited to artificially design TALE proteins interacting specifically with DNA sequences of interest to modify them by insertion, deletion, or other targeted rearrangements." (Bodnar et al)

When speaking of TALEs we must also understand the two most important DNA repair systems present in eukaryotic cells. These are Non-Homologous end-joining (NHEJ) and Homologous Recombination (HR). NHEJ produces DNA sequence changes such as deletion/insertion and substitutions in the target sequence. HR occurs when an exogenous sequence is introduced into the genome and recombination occurs to allow the incorporation of that gene into the target region. This has already been achieved through the use of ZFNs. But as mentioned before ZFNs still have major drawbacks. TALEs should be able to overcome these drawbacks and become true alternatives. A TALE is very specific and has many uses. It can be directed to a promoter region of a gene to allow for the induction of that gene, as we are doing. Or you can put an endonuclease at the end and cause a double stranded break to incur the gene editing as in TALENs.

TALEs have a very bright future ahead of them. My research is focused on optimizing the construction of those TALEs and to successfully activate the beta globin gene. While other papers have shown this to happen, the novelty in my research is the further use once we activate the beta globin gene. The use of the molecular beacons to specifically detect the sequences of nucleic acids is much understated because that will allow us to properly locate the exact location of the sequence in the cell that we want to repair. We will then be able to use TALENs to repair the beta globin gene in multiple

ways to try and fight sickle cell. While we are still far away from an actual animal model, significant advances are being made every day.

CHAPTER 3

MATERIALS AND METHODS

Some of the processes that we use in lab are PCR, Restriction Enzyme Digestion, Ligation, and Transformation of the E. coli, Colony Screening, Sequencing and Sequence Confirming, and Cell experiments. The methodology of the TALE construction has been developed over the past two years. The workflow is as follows:

1) Construct TALE Backbone
2) Construct Backbone with Fluorescence reporter (GFP, CFP, tdTomato, AmCyan)
3) Sequence confirm the TALEs
4) Analyze the TALEs in different Cell Lines (293T, K562, HBB::GFP K562)
5) Compile Data on the effectiveness of the TALEs in the cells

At this point in the project, work is being done to test the TALEs and different combinations of the TALEs to see the effectiveness in the up regulation of the beta globin gene. By testing the TALEs in the HBB::GFP K562 cells, we can see exactly how much the TALEs actually up regulate the beta globin, since the cells are specifically made to up regulate beta globin anyway.

CHAPTER 4

RESULTS

Construct TALE Backbone:

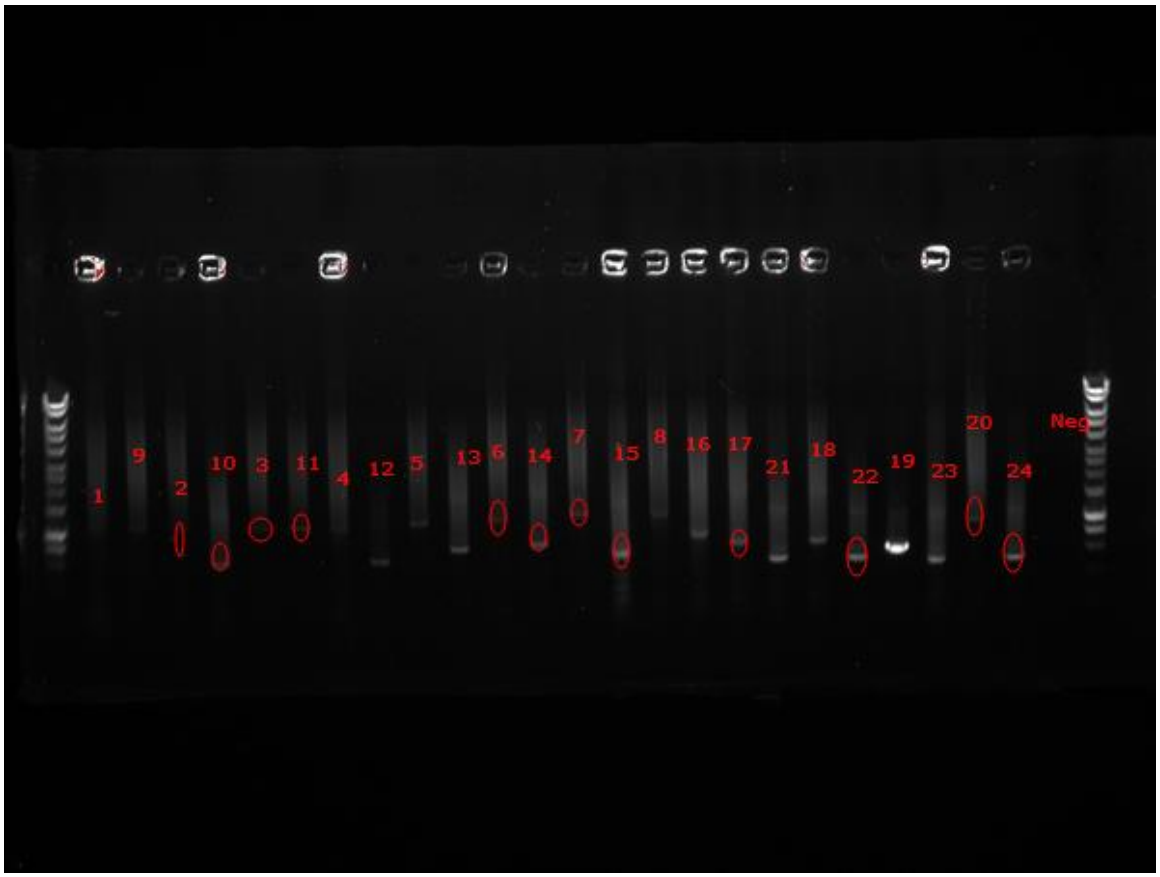

Figure 1. Colony Screen of TALE Backbone Construction

TALEs were constructed with different length of DNA sequence. TALEs were then Colony Screened with a Forward and Reverse primer. TALEs with circles are TALEs that are confirmed to be at the specific length that they needed to be.

Construct Backbone with Flourescent Reporters:

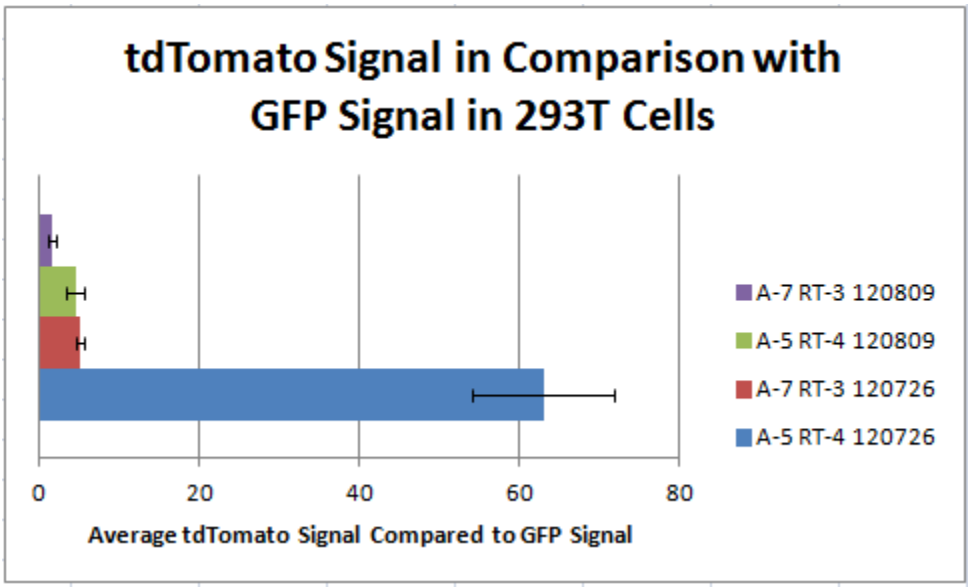

Figure 2. Comparison of tdTomato Signal and GFP Signal in 293T cells.

Above figure shows tdTomato signal compared against GFP signal in certain TALEs. TALE A-5 with RT-4 showed the highest signal when compared with the other TALEs and reporters. This shows that the signal was the brightest at that point.

CHAPTER 5

DISCUSSION/FUTURE WORK

TALEs are constructed with different reporters to see which reporters have the best specificity and sensitivity at the binding site. The tdTomato reporter shows the most promise. The reporter, tdTomato, will be used in different reporter systems to see which reporter system will show the best results. While we have used 293T, we will also try the same results in K562 cells to see if we can replicate the results. Different delivery strategies in the cell lines are being investigated. Recent work has shown that nucleofection with Fugene would be best in getting the TALEs inside the cells.

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